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Oxidative Modification of Low Density Lipoproteins by Human Polymorphonuclear Leukocytes

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Summary: Oxidatively modified low density lipoproteins are thought to play an important role in the generation of macrophage-derived foam cells in early atherosclerotic lesions. Cultured endothelial cells, monocytes, macrophages and smooth muscle cells can modify low density lipoproteins, either by a free radical mechanism or by the action of lipoxygenases. Previous studies demonstrated that activated human polymorphonuclear leukocytes can oxidize low density lipoprotein lipids. Stimulation of the cells with phorbol 12-myristate 13-acetate resulted in an increase both in superoxide anion production and in low density lipoprotein oxidation. The present results show that the oxidative modification of low density lipoproteins by human polymorphonuclear leukocytes can be inhibited by superoxide dismutase but not by the lipoxygenase inhibitor, (5,8,11,14)-eicosatetraynoic acid. The low density lipoproteins oxidized by polymorphonuclear leukocytes were recognized by the scavenger receptor of macrophages (P 388 cell line). It is proposed that the superoxide anion is an important factor in the the oxidative modification of low density lipoproteins induced by polymorphonuclear leukocytes, and that under conditions of increased oxidative metabolism in vivo, polymorphonuclear leukocytes can contribute to foam cell formation by a scavenger receptor-dependent process at lesion sites.

Introduction

The pathogenesis of atherosclerosis is complex and multifactorial. Hypercholesterolaemia is widely accepted as one of the major risk factors contributing to the development of coronary heart disease. From epidemiological studies (1, 2) and animal experiments (3, 4) it is clear that cholesterol is deposited in atherosclerotic plaques and that this cholesterol is mainly derived from plasma low density lipoproteins (5, 6). Recent evidence suggests an important role for the oxidative modification of low density lipoproteins in the pathogenesis of atherosclerosis. Incubation of low density lipoproteins with various cells such as endothelial cells, macrophages, human monocytes, and smooth muscle cells leads to an oxidatively modified form which is recognized by the scavenger receptor of macrophages, thus inducing foam cell formation (7). This type of modification is characterized by the peroxidation of low density lipoprotein lipids and the modification of the apolipoprotein B-100 by lipid peroxidation products (8, 9). It has been proposed that the oxidative modification of low density lipoproteins by cells can be mediated either by superoxide anions (10), or by the action of the 15-lipoxygenase enzyme (11, 12).

Leukocytes, both polymorphonuclear neutrophil leukocytes and monocytes, have been observed in the earliest atherosclerotic lesions (13, 14). When they encounter an appropriate ligand, these cells generate highly unstable oxygen species such as superoxide anions, hydrogen peroxide, hydroxyl radicals, and singlet oxygen (15). Increased leukocyte oxidative metabolism has been observed in hyperlipidaemic patients (16), and human polymorphonuclear leukocytes are known to stimulate peroxidation of low density lipoprotein lipids, as measured by the formation of thiobarbituric acid reactive substances and lipid hydroperoxides (17, 18). However, polymorphonuclear

[1]) This work is part of the doctoral thesis of A. Brandes.

leukocytes also contain the 15-lipoxygenase enzyme (19). Modification of the apolipoprotein B-100 by neutrophil elastase has also been reported and it was suggested that such elastase-modified low density lipoproteins may contribute to foam cell formation (20). However, these elastase-modified low density lipoproteins are recognized by the low density lipoprotein receptor and not by the scavenger receptor of macrophages.

The present study was designed to clarify the mechanism of the oxidative modification of low density lipoproteins by polymorphonuclear leukocytes and to determine whether such modified low density lipoproteins are recognized by the scavenger receptor of macrophages.

Materials and Methods

Preparation of cells

Human polymorphonuclear leukocytes were isolated by density gradient centrifugation (21) using a 4 + 1 (by vol.) mixture of neutrophil isolation medium (NIM; Los Alamos Diagnostics, Los Alamos, NM, USA) and Lymphoprep (Nycomed, Oslo, Norway). Five ml venous blood anticoagulated with 10 000 U/l Na-heparin were layered over an equal volume of the NIM/Lymphoprep mixture in sterile plastic tubes and centrifuged at 400 *g* for 40 min at 4 °C. Polymorphonuclear leukocytes were removed by aspiration and washed 3 times with *Hank*'s Balanced Salt Solution (GIBCO, Paisley, UK). After the last wash erythrocytes were lysed for 30–60 seconds with H_2O and the polymorphonuclear leukocyte suspension was washed again with *Hank*'s Balanced Salt Solution. The resulting pellet was resuspended in serum-free *Ham*'s F-10 medium (GIBCO, Paisley, UK) containing 100 × 10^3 U/l penicillin and 100 mg/l streptomycin. Cell numbers and purity were determined with an automatic cell counter (Technicon H 1, Bayer Diagnostic Co, Tarrytown, NY, USA) using the differential counting mode. Cell preparations contained 98% polymorphonuclear leukocytes, 2% lymphocytes, but no contamination by monocytes. Unless indicated otherwise, cell numbers were adjusted to 3 × 10^9/l and plated in serum-free *Ham*'s F-10 medium (GIBCO, Paisley, UK) on 35 mm plastic cell culture dishes (Nunc, Roskilde, DK). Cells were allowed to settle for 15 min, then used for modification studies without further washing.

For degradation experiments the murine macrophage cell line P 388 D (Paesel-Lorey GmbH, Frankfurt, Germany) was used. Cells were plated on 35 mm culture dishes in RPMI 1640 medium (GIBCO, Paisley, UK) containing fetal calf serum (volume fraction 0.1) and antibiotics (100 × 10^3 U/l penicillin, 100 mg/l streptomycin). At confluence, cells were washed twice with serum-free RPMI 1640 medium, then used for degradation studies with low density lipoproteins.

Isolation and modification of low density lipoproteins

Low density lipoproteins (d = 1.019–1.063 kg/l) were isolated by ultracentrifugation from the plasma of healthy donors using an established protocol (22). After isolation, low density lipoproteins were extensively dialysed against a Tris-HCl buffer (5 mmol/l, pH 7.4) containing 1 mmol/l EDTA. The cholesterol, triacylglycerol, phospholipid and protein content of the low density lipoprotein preparation were determined. Low density lipoproteins were labelled with ^{125}I by the iodine monochloride method (23). Before modification, low density lipoproteins were dialysed against a nitrogen-saturated Tris-HCl buffer (5 mmol/l, pH 7.4) free of EDTA and diluted to a protein concentration of 0.1 g/l. Incubations were performed for 24 h in 35-mm culture dishes with serum-free *Ham*'s F-10 medium (2 ml) in the presence of either polymorphonuclear leukocytes or 10 µmol/l $CuSO_4$ (37 °C, 95% O_2, 5% CO_2). In some incubations, cells were activated with 6.25 nmol/l phorbol 12-myristate 13-acetate (Sigma Chemical Co., St. Louis, MO, USA). Cell integrity was monitored visually by phase contrast light microscopy and trypan blue exclusion. The protease inhibitor, aprotinin (0.1 g/l) (Sigma Chemical Co., St. Louis, MO, USA), was added to the incubations to prevent non-specific degradation of the apolipoprotein B-100. Low density lipoproteins were acetylated with acetic anhydride and saturated sodium acetate as described (24).

Analytical techniques

The extent of lipid peroxidation in the modified low density lipoproteins was quantified by measuring thiobarbituric acid-reactive substances, and expressed as malondialdehyde equivalents (8). The electrophoretic mobility of the modified low density lipoproteins was tested by agarose gel electrophoresis (25) using a commercially available kit (Lipidophor, Immuno GmbH, Vienna, Austria). The ability of macrophages to degrade the modified low density lipoproteins was determined as follows: 10 µg of ^{125}I-labelled low density lipoproteins (native or modified) were added to macrophages in 35-mm cell culture dishes in 1 ml of RPMI 1640, and incubated at 37 °C for 5 h. The medium was then removed and analysed for non-iodide trichloroacetic acid-soluble radioactivity as described elsewhere (24). Control dishes without macrophages were run and specific degradation was calculated by subtracting these cell-free controls from incubations in the presence of macrophages. Competition experiments were performed using a tenfold excess of unlabelled low density lipoproteins.

Protein was determined by the *Lowry* method (26) using serum albumin as a standard. Superoxide anion production was determined by measuring superoxide dismutase[2]) inhibitable cytochrome c reduction, using the end point procedure described by *Markert* et al. (27). Cholesterol, triacylglycerols and phospholipids were determined by established standard methods in our routine clinical chemistry laboratory.

Inhibitors

5,8,11,14-Eicosatetraynoic acid was purchased from Cayman Chemical, Ann Arbor, MI, USA. Butylated hydroxytoluene, mannitol, catalase[2]) and superoxide dismutase were from Sigma, Chemical Co., St. Louis, MO, USA. Butylated hydroxytoluene and eicosatetraynoic acid were dissolved in ethanol while all other inhibitors were dissolved in *Ham*'s F-10 medium (GIBCO, Paisley, UK). Inhibitors were added to the incubations in 20 µl aliquots before the start of incubations. Control dishes were incubated under identical conditions but in the absence of inhibitors. Controls without cells or copper ions were also run. Superoxide dismutase was heat inactivated by incubating the enzyme at 90 °C for 10 min in a shaking water bath.

Statistics

Statistical analysis was performed using the *Wilcoxon* test for matched pairs.

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[2]) Enzymes
Catalase: Hydrogen-peroxide: hydrogen-peroxide oxidoreductase (EC 1.11.1.6)
Superoxide dismutase: Superoxide: superoxide oxidoreductase (EC 1.15.1.1)

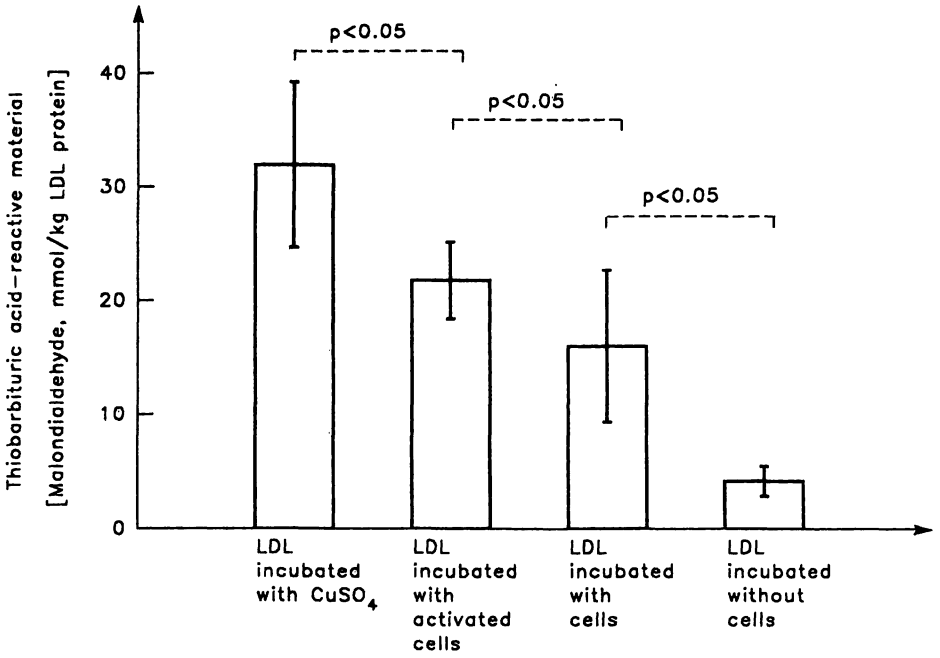

Fig. 1. Oxidation of low density lipoprotein (LDL) preparations (n = 5) by copper ions, polymorphonuclear leukocytes and activated polymorphonuclear leukocytes. Cells were activated with phorbol 12-myristate 13-acetate. Experimental conditions as described in Materials and Methods.

Results

Incubation of low density lipoproteins (protein concentration 0.1 g/l) for 24 h in the presence of freshly isolated human polymorphonuclear leukocytes (3×10^6 cells per dish) produced 16.0 ± 6.9 mmol malondialdehyde equivalents per kg low density lipoprotein protein (n = 5), whereas control incubations without cells produced 4.2 ± 1.4 mmol malondialdehyde equivalents per kg low density lipoprotein (n = 5; $p < 0.05$). Lipid peroxidation was dependent on the cell number: 5×10^5 polymorphonuclear leukocytes yielded 7 mmol malondialdehyde equivalents per kg low density lipoprotein protein, whereas 1×10^6 cells yielded 9.5 mmol malondialdehyde equivalents per kg low density lipoprotein. Addition of 6.25 nmol/l phorbol 12-myristate 13-acetate to 3×10^6 polymorphonuclear leukocytes caused a further statistically significant increase of peroxidation of low density lipoprotein lipids (21.7 ± 3.4 mmol malondialdehyde equivalents per kg low density lipoprotein protein; n = 5; $p < 0.05$) (fig. 1). Acceleration of lipid peroxidation by activated cells was apparent beyond 6 hours of incubation (fig. 2). The enhanced oxidation of low density lipoprotein lipids by acti-

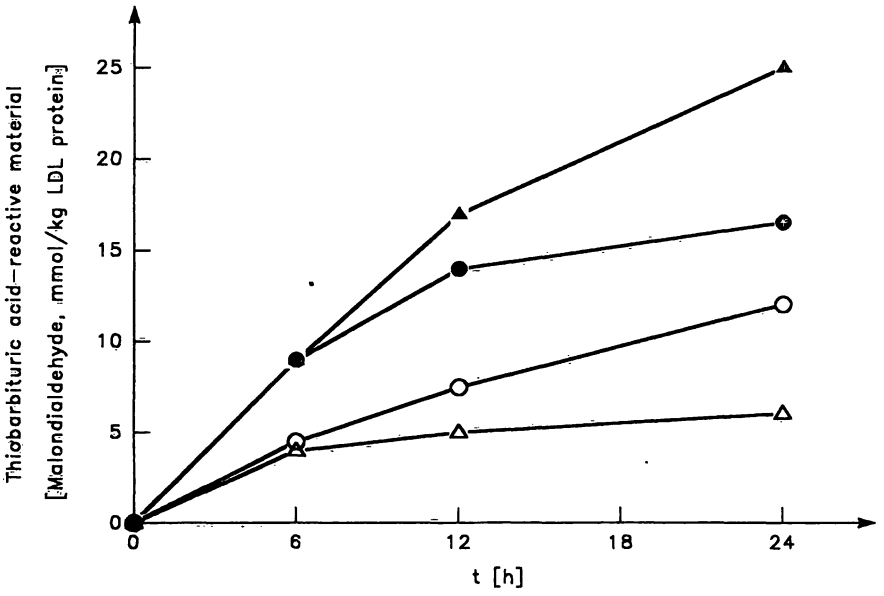

Fig. 2. Time course of thiobarbituric acid reactive substance formation during incubation of a low density lipoprotein (LDL) preparation with polymorphonuclear leukocytes (open circles), activated polymorphonuclear leukocytes (filled circles), copper ions (filled triangles), and control incubations without cells or copper ions (open triangles). Incubation conditions are as described in Materials and Methods. Mean values of duplicate determinations from one experiment.

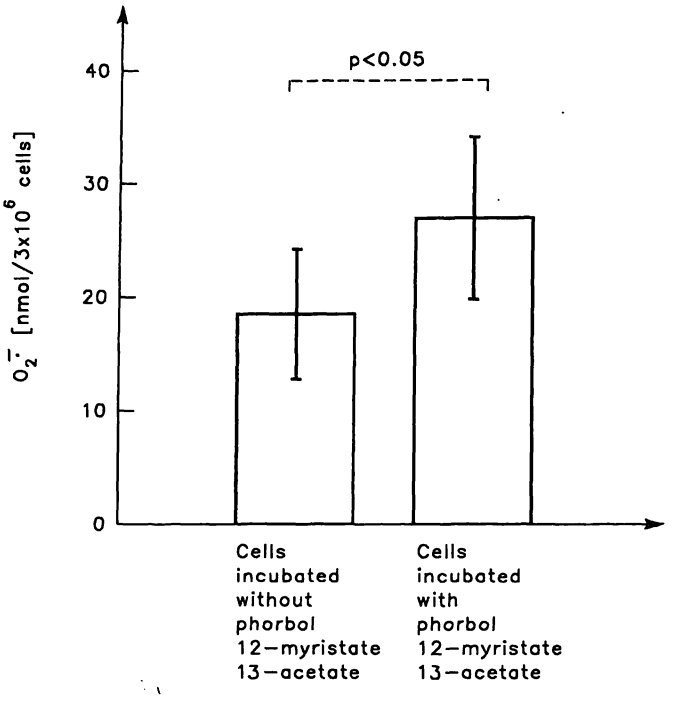

Fig. 3. Superoxide anion production by polymorphonuclear leukocytes (n = 5) in the presence and absence of phorbol 12-myristate 13-acetate. Experimental conditions as described in Materials and Methods.

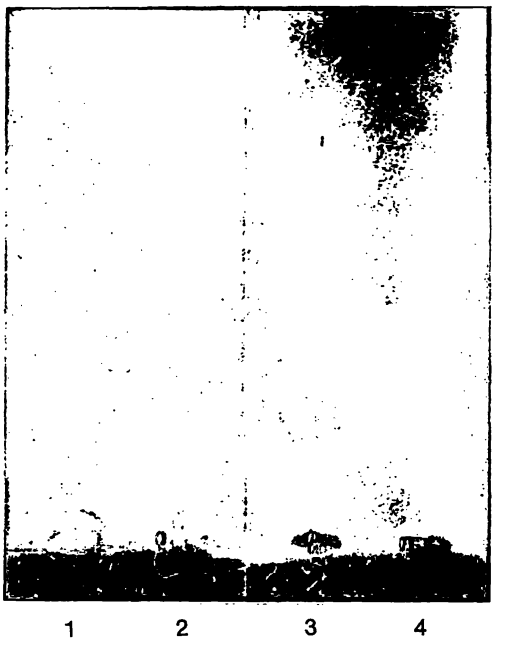

Fig. 4. Agarose gel electrophoresis of a low density lipoprotein (LDL) preparation incubated for 24 h in the presence of activated polymorphonuclear leukocytes (lane 1), non-activated polymorphonuclear leukocytes (lane 2), native LDL (lane 3), and LDL incubated without cells (lane 4). Incubation conditions as described in Materials and Methods.

vated polymorphonuclear leukocytes was accompanied by an increased production of superoxide anions (fig. 3).

Modification of apolipoprotein B-100 by lipid peroxidation products was shown by agarose gel electrophoresis. Low density lipoprotein particles incubated for 24 h in the presence of polymorphonuclear leukocytes showed an increased negative charge compared with native low density lipoprotein particles incubated without cells (fig. 4). Furthermore, macrophage degradation of low density lipoproteins modified by polymorphonuclear leukocytes was enhanced (tab. 1). The accelerated lipid peroxidation achieved by stimulation of the cells with phorbol 12-myristate 13-acetate also affected the degradation of these low density lipoprotein particles by macrophages (tab. 1). Aprotinin, which was added to prevent degradation of apolipoprotein B-100 by proteases, had no effect on the degradation of modified low density lipoproteins by macrophages (tab. 1).

Macrophage degradation of low density lipoproteins modified by polymorphonuclear leukocytes was inhibited by a 10-fold excess of unlabelled acetyl- or copper-modified low density lipoproteins. Unlabelled acetylated low density lipoproteins and low density lipoproteins oxidized by copper ions displaced 60% and 80%, respectively, of the ^{125}I-labelled low density lipoproteins which had previously been incubated in the presence of polymorphonuclear leukocytes for 24 h (fig. 5).

The effect of various inhibitors and radical scavengers on the oxidation of low density lipoproteins by polymorphonuclear leukocytes is shown in table 2. Addition of 30 kU/l superoxide dismutase resulted in a marked inhibition of peroxidation of low density lipoprotein lipids in both activated and non-activated

Tab. 1. Macrophage degradation of a low density lipoprotein (LDL) preparation previously incubated in the presence of polymorphonuclear leukocytes

Sample	n	Degradation by macrophages (g/5 h · kg cell protein)
LDL incubated in the absence of cells	7	0.30 ± 0.07
LDL incubated in the presence of $CuSO_4$	5	1.40 ± 0.11*
LDL incubated in the presence of $CuSO_4$ and aprotinin	3	1.36 – 1.43*+
LDL incubated in the presence of cells and aprotinin	7	0.98 ± 0.23*
LDL incubated in the presence of activated cells plus aprotinin	7	1.29 ± 0.35**

Values are means ± SD or range (+) from the indicated number of experiments. For cell activation, phorbol 12-myristate 13-acetate was used as described in Materials and Methods.

* Significantly different from low density lipoproteins incubated in the absence of cells ($p < 0.05$)

** Significantly different from low density lipoproteins incubated in the presence of cells and aprotinin without phorbol myristate acetate ($p < 0.05$)

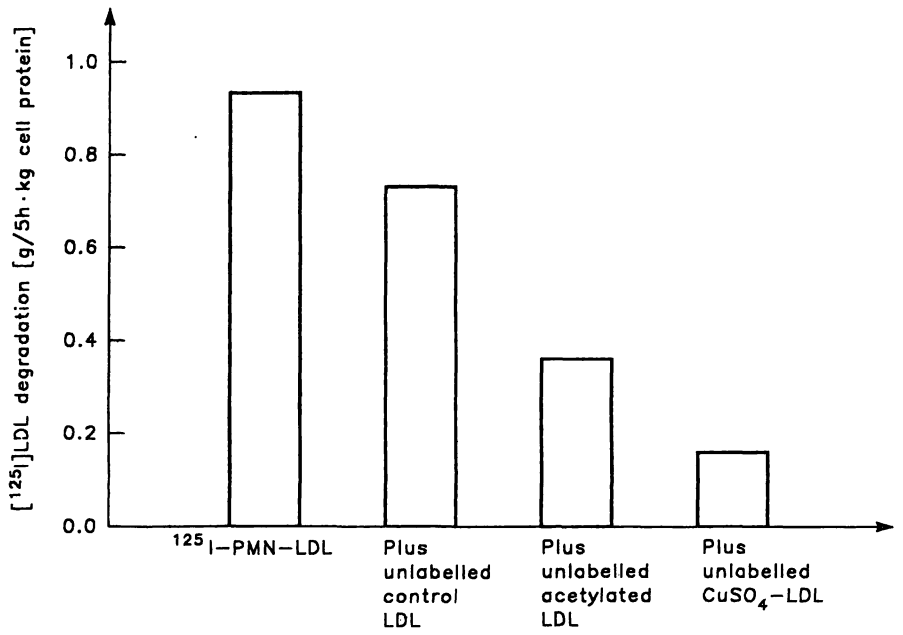

Fig. 5. Macrophage degradation of a labelled low density lipoprotein preparation previously incubated in the presence of polymorphonuclear leukocytes (^{125}I-PMN-LDL). Displacement by unlabelled LDL incubated without cells (control LDL), by unlabelled acetylated LDL, or by unlabelled LDL previously incubated in the presence of copper ions ($CuSO_4$-LDL). Experimental conditions as described in Materials and Methods. Mean values of duplicate determinations from one experiment.

cells. Most of the inhibitory effect was abolished after heating of the enzyme to 90 °C for 10 min. Similar effects were observed with 40 µmol/l of the non-specific general antioxidant, butylated hydroxytoluene. The lipoxygenase inhibitor, eicosatetraynoic acid, was without effect on the oxidation of low density lipoproteins by human polymorphonuclear leukocytes, as was catalase (110 kU/l) and the hydroxyl radical scavenger, mannitol (25 mmol/l).

Tab. 2. Effect of different inhibitors on the oxidation of a low density lipoprotein (LDL) preparation by polymorphonuclear leukocytes.

Inhibitor added	Thiobarbituric acid-reactive materials (Malondialdehyde, mmol/kg LDL protein)	
	Cells plus LDL	Activated cells plus LDL
No inhibitor	10.3	18.4
Butylated hydroxytoluene (40 µmol/l)	1.6	1.8
Superoxide dismutase (30 kU/l)	1.8	6.0
Superoxide dismutase (heat inactivated)	n. d.	14.1
Catalase (110 kU/l)	n. d.	16.9
Eicosatetraynoic acid (20 µmol/l)	8.7	17.4
Mannitol (25 mmol/l)	10.0	18.2
LDL incubated without cells and without inhibitor	2.2	

Values are means of duplicate determinations from one representative experiment. For cell activation, phorbol 12-myristate 13-acetate was used as described in Materials and Methods. n. d. = not determined.

Discussion

Oxidative modification of low density lipoproteins has been implicated in the generation of macrophage-derived foam cells (7–12), a hallmark of the early atherosclerotic lesion (14, 24). Once the endothelial lining of the vessel wall has been damaged, the progression of atherosclerosis can be considered as a chronic inflammatory process (13).

Polymorphonuclear leukocytes are found in the subendothelial space at sites of arterial damage in experimental animals (13). These cells contain lipoxygenase enzymes and release oxygen radicals upon encounter with an appropriate ligand (15, 19). They have been shown to oxidize low density lipoprotein lipids, making them cytotoxic (17), and to alter low density lipoproteins by the action of elastase, resulting in their increased uptake by macrophages via the classical low density lipoprotein receptor (20). In contrast, our results show that incubation of low density lipoproteins with human polymorphonuclear leukocytes leads to oxidatively modified low density lipoproteins which are recognized by the scavenger receptor of macrophages. Scavenger receptor-mediated uptake of modified low density lipoproteins must be considered more important for the development of artheroscler-

otic plaques in vivo, since monocytes lose most of their low density lipoprotein receptor activity upon transformation to macrophages, which then express scavenger receptor activity (24). The observation that low density lipoproteins modified by human polymorphonuclear leukocytes are displaced less efficiently by acetylated low density lipoproteins than by low density lipoproteins modified by copper ions, confirms findings of *Sparrow* et al. concerning the heterogeneity of the scavenger receptor with respect to different ligands (28).

The present investigations strongly suggest a role of superoxide anions in the oxidative modification of low density lipoproteins by polymorphonuclear leukocytes and exclude a lipoxygenase-mediated oxidation mechanism for the following reasons:

i) oxidation of low density lipoproteins was inhibited by superoxide dismutase

ii) eicosatetraynoic acid was without effect on the oxidation of low density lipoproteins

iii) stimulation of superoxide anion release increased the modification of low density lipoproteins

Phorbol 12-myristate 13-acetate is a direct activator of protein kinase C and therefore selectively stimulates the formation of superoxide anions. It bypasses the activation of eicosanoid formation in human polymorphonuclear leukocytes (29).

This mechanism of low density lipoprotein modification in polymorphonuclear leukocytes is in contrast to macrophages and endothelial cells where 15-lipoxygenase activity has been suggested to be responsible for the oxidative modification of low density lipoproteins (11, 12).

Abdalla et al. (18) reported peroxidation of low density lipoprotein lipids by polymorphonuclear leukocytes activated with substances which are known to increase leukotriene production (29). However, they did not employ lipoxygenase inhibitors to exclude a contribution of lipoxygenase enzymes to the modification of low density lipoproteins by human polymorphonuclear leukocytes. The authors emphasized the requirement for iron to initiate peroxidation of low density lipoprotein lipids. In their system, however, containing activated polymorphonuclear leukocytes and ferritin, they generated mainly lipid hydroperoxides, with only very small quantities of aldehydes which are required for the modification of the apolipoprotein B-100 and therefore for foam cell formation (9). We used the protocol originally published by *Henriksen* et al. (30) to oxidize low density lipoproteins; this protocol uses the iron-containing *Ham*'s F-10 cell culture medium. With his approach we were able to generate 10 times more malondialdehyde equivalents than reported by *Abdalla* et al. (18). Since catalase and mannitol did not influence the oxidative modification of low density lipoproteins by polymorphonuclear leukocytes it is unlikely that other reactive oxygen species such as hydrogen peroxide or hydroxyl radicals contribute to the oxidation process.

Oxidation of low density lipoproteins by polymorphonuclear leukocytes was observed even in the absence of a stimulus. *Nathan* reported that the adhesion of polymorphonuclear leukocytes to plastic surfaces is sufficient to activate polymorphonuclear leukocytes at a low rate (31). This is supported by the present investigation using adherent cells for low density lipoprotein modification, which had already generated superoxide anions at a low rate in the absence of a stimulus.

It is concluded that an increase in the release of superoxide anion from polymorphonuclear leukocytes, as may occur at lesion sites in vivo, can accelerate oxidative modification of low density lipoproteins. Possible activators in vivo are immune complexes (32). Autoantibodies directed against oxidatively modified low density lipoproteins have recently been reported in vivo (33). Therefore, polymorphonuclear leukocytes may contribute to foam cell formation in vivo as do other cells found in atherosclerotic lesions, such as endothelial cells, macrophages, smooth muscle cells, and monocytes.

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